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Genetic detection of *Theileria annulata* from Bos taurus and *Bubalus bubalis* during health surveillance at marsh breeders' farm in Basra Marshes, Iraq

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Abstract

In the context of health monitoring of a group of cattle and buffalo farms in marsh and swamp areas, these animals displayed evident clinical signs of nutritional deficiency in addition to symptoms that included high temperature (41 °C or higher), swollen lymph nodes, diarrhea, anemia, weakness, and decreased appetite. And, in some cases, cough. The presence of ticks in various areas of the body, especially the edges of the ear, the neck area and the area beneath the tail, indicated the possible presence of Theileriosis, a parasitic infection. Random blood samples were collected from sixteen individuals. Subsequently, DNA was extracted from these samples and Polymerase Chain Reaction (PCR) was utilized to amplify the small subunit of the 18S rRNA gene, which is highly specific for the detection of Babesia/Theileria species. The PCR procedure employed the GF (5'-G(C/T) (C/T) TTGT AAT TGG AAT GAT GG-3') and GR (5'-CCA AAG ACT TTG ATT TCT CTC-3') primers. The results were then compared to international isolates via an analysis of genetic nucleotide sequences using the Basic Local Alignment Search Tool (BLASTn) algorithm, available at the National Center for Biotechnology Information (NCBI). This analysis unveiled a significant genetic resemblance between the 18S rRNA gene sequences and *T. annulata* species, suggesting the presence of this parasite. As a consequential outcome of this study, it has been established that *Bos taurus* and *Bubalus bubalis*, can be a new host for *T. annulata*, particularly in the southern regions of Iraq.

Keywords: *Theileria annulata*، 18S rRNA gene, *Bos taurus, Bubalus bubalis*, Iraq.

Introduction

Ticks are hematophagous ectoparasites that are considered obligate. They transmit a greater variety of pathogens to humans and animals through blood-feeding compared to any other arthropods (1). *Theileria annulata*, which causes tropical theileriosis, is transmitted to bovines by ixodid ticks (2). According to (3),, Theileriosis is a potentially fatal disease that leads to economic losses in susceptible cattle worldwide. While it can affect cattle of all ages, those that are stressed, heavily pregnant, or lactating are at the highest risk due to reduced immunity (4). Clinical signs of tropical theileriosis include fever, slight nasal discharge, ocular symptoms, anorexia, and salivation, enlargement of superficial lymph nodes, acute anemia, respiratory distress, jaundice, and asphyxia-induced death (5). The most common lesions associated with *Theileria* infection involve enlargement of lymph nodes and spleen, presence of Koch's bodies (Koch's postulates) in multiple organs, pulmonary emphysema, subcutaneous and intramuscular hemorrhages, excessive pericardial and pleural fluids, and enlargement of the liver (6, 7). *Theileria*, a blood protozoan parasite, is found worldwide. The simplest diagnostic tests include the clinical signs, Giemsa-stained blood or tissue smears, to detect macroschizonts or periplasms in infected animals (8, 9) These tests are effective in detecting active infections, as animals often have high parasite (10).. In cases with acute symptoms, the diagnosis of Theileriosis primarily relies on microscopic examination of blood smears stained with Giemsa (11, 12). (13) state that serological tests, such as enzyme-linked immunosorbent assay (ELISA), and molecular biology techniques are more accurate and confirmatory laboratory methods for diagnosing theileriosis. The current study aims to monitor the health of a group of buffalo and cow at a cattle breeder's farm to early detect significant injuries that may pose a threat to public health.

Ethics approval and consent to participate

The welfare of animals in the Republic of Iraq was diligently upheld in accordance with the country's established laws, guidelines, and policies on animal welfare. These regulations were officially endorsed by the University of Basrah, as indicated by Permit reference number 3/7/13302/2019. Furthermore, all activities within fishing areas designated for human consumption were conducted with the necessary authorizations from the Directorate and with the full support of the Department of Veterinary Microbiology and Parasitology.

Materials And Methods

Samples collection

The research was conducted during the July of the year 2019 in the marshes of Basrah Province, located in the Southern part of Iraq. Sixteen blood samples were collected from both the jugular and ear veins of animals, as per the guidelines provided in reference (14). The blood samples were obtained from eight cattle (*Bos taurus*) and eight buffalo (*Bubalus bubalis*). EDTA tubes were used for blood collection, and subsequently, the samples were frozen for

preservation. Staining of blood smear with Giemsa stain and examined under 100x magnification with aid oil emersion.

DNA extraction

DNA extraction was carried out from the whole blood samples following the instructions provided in the leaflet of the commercial kit (Geneaid DNA extraction kit-Bioneer). To detect *Babesia/Theileria* spp., the amplification fragment of the 18S rDNA was targeted using specific primers, namely GF (5'-G(C/T) (C/T) TTGT AAT TGG AAT GAT GG-3') and GR (5'-CCA AAG ACT TTG ATT TCT CTC-3') (15,16). The PCR reaction was performed in a total volume of 15 µl, containing 30 ng of template DNA, 7.5 µl of 2× Master mix (Fermentas), 0.5 µl of each forward and reverse primer (10 pmol/µl), and 5.5 µl of nuclease-free water. The PCR program included the following steps: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. The PCR products, representing partial amplification (approximately 500 bp) of the 18S rRNA gene of *Theileria* spp., were visualized through agarose gel electrophoresis (1.5%) under UV light. SYBR Safe (Gel Green) was used to stain the DNA. A total of 10 µl of DNA was loaded into the wells of a 0.2% agarose gel. The cathode and anode were connected to the respective poles, and electrophoresis was carried out at 80 volts for 40 minutes. The gel was run until the bromophenol blue dye had migrated, and the resulting bands were examined using a UV trans illuminator. All reference sequences used in the study were obtained from GenBank through the BLASTn algorithm available at https://blast.ncbi.nlm.nih.gov. Multiple sequence alignments of the DNA sequences were performed using MUSCLE v. 3.8.31. The evolutionary history was inferred using the neighbor-joining method, and the evolutionary distances were computed using the maximum composite likelihood method (17, 18). The 18S rRNA gene sequence has been submitted to GenBank.

Results

In this study, the presence of the parasite was detected by clinical signs in infected animals, were observed some signs, including weakness and emaciation, enlarged lymph nodes that appeared as oval or round rods, elevate of temperature to 41 degrees Celsius or higher, diarrhea, anemia, loss of appetite, and occasional cough. In addition to the presence of ticks on the neck and around the ear area and beneath the tail. To confirm the causative agents, a blood of suspected animals was examined and stained using Giemsa stain. The morphological examination indicated that the red blood cells were infected with the *Theileria anuulata*, as shown in Fig. (1).

In terms of molecular detection, the parasite was identified through PCR analysis, which yielded specific DNA fragments of approximately 500 base pairs in length. This is illustrated in Figure 2, where the PCR products could be seen separated on an agarose gel.

Phylogenetic analysis

The research revealed the existence of a particular species based on the findings from DNA sequencing. The nucleotide sequences

of the 18S rRNA gene, partially amplified from one of the blood-positive samples, were recorded in GenBank with the accession number ON854089.1. These sequences exhibited a complete match of 479/479 (100%) with the isolate from the present study, as well as with the same species found in various hosts and geographical regions.

The 18S rRNA gene isolates were subjected to phylogenetic analysis using the neighbor-joining method with the Kimura distance calculation. The results obtained were highly consistent (refer to Figure 3). *T. annulata*, along with the Algeria species strains (GenBank:MH 327773), formed a monophyletic group, indicating a shared common ancestor.

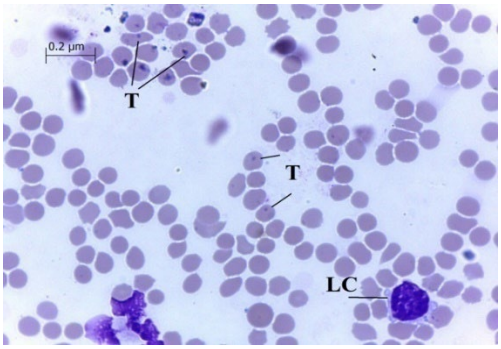

Figure1. Red blood cells infected with *T. annulata* (T), LC: Lymphocytes, staining with Giemsa stain and examination under (100 x) magnification

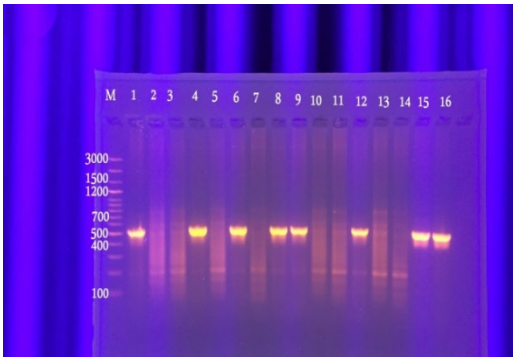

Figure 2: Agarose gel electrophoresis (1.5%) for the PCR products shows partial amplification (approximately 500 bp) of the 18S rRNA gene of T. annulata DNA ladder (3000 bp), lanes no. 1, 4, 6, 8, 9, 12, 15, are the positive samples, and lanes 2, 3, 5, 7, 10, 11, 13, and 14 are the negative samples.

Table 1. The identity percentage of *Theileria* species of the current study and those isolated from different countries and provided by NCBI/ GenBank.

Current isolate	Identity% references	Organism	Reference	Geographical area	Host
ON854089.1	479/479(100%)	*T. annulata*	MN907457.1	China	Tick
	479/479(100%)	*T. annulata*	OM273908.1	Egypt	Camel
	479/479(100%)	*T. annulata*	OM140998.1	China	Tick
	479/479(100%)	*T. annulata*	OM140997.1	China	Tick
	479/479(100%)	*T. annulata*	OM066190.1	Turkey	Cattle

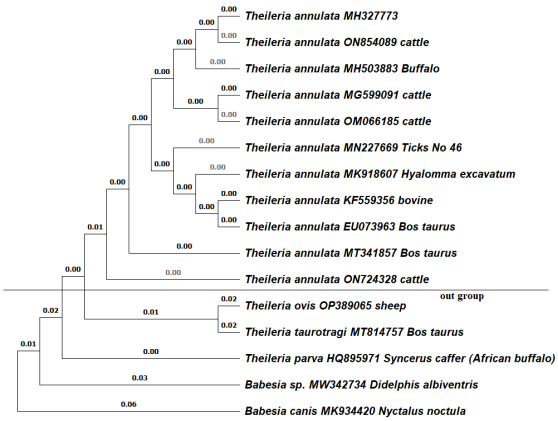

Figure 3: Cladogram of Neighbor-joining analysis of the 18S rRNA gene sequences showing the phylogenetic relationship of the Theileria spp.

Discussion

In a previous study, (19) emphasized the importance of buffaloes in the spread of theileriosis, as they serve as a reservoir host for the species (20). The parasite *T. annulata* is particularly endemic in buffaloes in the Asian region (21). What is interesting is that these hosts generally exhibit mild non-acute disease symptoms, making diagnosis difficult, while the parasite poses a significant threat to cattle (22). Furthermore, buffaloes can carry the parasite for up to two years without reinfection and without showing any disease symptoms, playing a crucial role as a source of bovine theileriosis (23,24) Additionally, disease incidence is influenced by several factors, including the geographical location of infection, the intensity of infection in host animals, and the animals' immunity (25).

The affected animals, especially cows, experienced clear clinical symptoms. It should be noted that higher infection rates may be observed with larger sample sizes. Failure to implement biosecurity measures

for infected samples can worsen the animals' condition and exacerbate the incidence of infections, which may not be confined to specific regions. The study's findings revealed immune deficiency and malnutrition in infected animals, indicating the disease's impact on the animals. According to [26], certain cattle breeds are more susceptible to *T. annulata* infection due to genetic variations, although there are shared protective components across all breeds. This study shed light on the high prevalence of blood parasite infections in cattle, which are often overlooked due to cattle not being considered a significant source of infection.

Accurate diagnosis of these parasites typically relies on clinical symptoms and the observation of morphological features of periplasms within red blood cells in stained smears. However, relying solely on these methods can lead to significant diagnostic errors were used to detect *Babesia/Theileria* spp. by amplifying a portion of the 18S rDNA (15, 16), and all nucleotide sequences matched *Theileria* infection. The lack of detection of Babesia spp. may be due to the parasite level in the peripheral blood being below the detection limit of selective PCR. This result aligns with the observations of (27) in their study. The results demonstrated that the classified isolate, which exhibited similar results to *T. annulata*, formed a common lineage and a single unique group with (GenBank: MH327773) strains from Algerian species isolated from ticks and mammal blood samples in northeastern Algeria. The group received strong support (bootstrap value = 0.00), indicating that our type and the Algerian type may have originated from a common ancestor, with several factors potentially contributing to the spread of this common lineage worldwide.

In conclusion, understanding the epidemiological distribution of pathogenic parasites in animal herds can lead to the development of strategies to control tropical theileriosis in Iraq. The use of genetic markers is the most effective method for diagnosing the studied species, in addition to the results of other traditional methods. Disease prevalence depends on the geographical area of infection, the intensity of infection in carrier hosts, and animal immunity (28, 29). Affected herds often suffer from noticeable clinical symptoms, and it is important to note that infection rates may be higher with larger sample sizes. Failure to implement biosecurity measures for infected

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Conflict Of Interest

The authors declare no conflict of interest.

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الكشف الجيني عن *Theileria annulata* من البقر Bos *taurus* والجاموس *Bubalus bubalis* خلال المراقبة الصحية في مزرعة مربي الأهوار في أهوار البصرة، العراق

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الخلاصة

في سياق المراقبة الصحية لمجموعة من المزارع لتربية الابقار والجاموس في مناطق الاهوار والمستنقعات. أظهرت الحيوانات المصابة علامات واضحة لنقص التغذية وأعراض سريرية حادة. شملت هذه الأعراض حمى مرتفعة (41 درجة مئوية أو أعلى)، وتضخم العقد اللمفية، والإسهال، وفقر الدم، والضعف، والفقدان في الشهية، وفي بعض الحالات، السعال. وكانت هناك علامات أيضًا على انتشار القراد في مناطق مختلفة من الجسم، حيث تأثرت الأماكن الواقعة على حواف الأذن ومنطقة العنق والمنطقة تحت الذيل بشكل أكبر، مما يشير إلى وجود إصابة بداء الثيليريات، وهو طفيلي .تم جمع عينات عشوائية من الدم من ستة عشر فردًا. بعد ذلك، تم استخراج الحمض النووي (DNA) من هذه العينات وتم استخدام تفاعل سلسلة البوليميريز (PCR) لتضخيم الوحدة الصغيرة لجين S rRNA18، والذي يعتبر محددًا لاكتشاف أنواع *Theileria /Babesia*. تم إجراء عملية PCR باستخدام الأساسيات ('GF (5'-G(C/T) (C/T) TTGT AAT TGG AAT GAT GG-3' و GR (5'-CCA AAG ACT TTG ATT TCT CTC-3').ثم تمت مقارنة النتائج المحصلة مع العينات الدولية من خلال تحليل التسلسل الوراثي للنوكليوتيدات باستخدام خوارزمية Basic Local Alignment Search Tool (BLASTn) المتوفرة في المركز الوطني للتكنولوجيا الحيوية (NCBI).أظهر هذا التحليل تشابها وراثيا كبيرا بين تسلسلات جين S rRNA 18 وسلالة T. annulata ، مما يشير إلى وجود هذا الطفيل .نتيجة لهذه الدراسة، أن الابقار والجاموس، يمكن أن يكون مضيفًا جديدًا لسلالة *T. annulata* ، وخصوصا في المناطق الجنوبية من العراق.

الكلمات المفتاحية: *Theileria annulata*، الجين الرايبوزي 18S، الابقار والجاموس، العراق.